REMARKS

Claims 1-6, 9-10 and 17-23 are pending. Claims 7 and 8 were previously canceled and claims 11-16 were previously withdrawn. Amendment of claims herein is made without abandonment of the original subject matter; applicants reserve the right to pursue claims of the original or similar scope in a duly filed continuing application.

Claim 1 has been amended to recite that the gene or gene fragment carried by the vector is under the control of an eukaryotic promoter. Support for this amendment is found, e.g., at page 4, line 22, as well as page 13, line 6 and page 14, line 20 of the application as originally filed. Claims 9 and 23 have been amended as requested by the examiner to clarify the recited gene. Claims 17 and 18 have been amended to recite that the gene or gene fragment encodes a polypeptide capable of inducing an antibody response and a T-cell response. Support for the recitation of "capable of inducing an antibody response" is found at page 7, lines 12-13, and support for "capable of inducing a T cell response" is disclosed in the headline in the middle of page 5 of the specification.

Thus, all claim amendments herein find basis in the original specification and no new matter has been introduced.

OUTSTANDING REJECTIONS

The rejections of claims 9 under 35 U.S.C. § 112, second paragraph for asserted indefiniteness was maintained. Claims 1-6, 9, 10 and 17-23 were also newly rejected for asserted indefiniteness.

Claim 1 and all claims dependent therefrom and claim 18 were rejected under 35 U.S.C. § 112, first paragraph as assertedly containing new matter.

Claims 1-3, 9-10 and 17-23 were rejected under 35 U.S.C. § 102 in view of Yang et al., J. Immunol. 145:2281-2285, 1990 ("Yang"); Tite et al., Immunology 70:540-546, 1990 ("Tite"); Verma et al., Vaccine 13:142-150, 1996 ("Verma"); and Fouts et al., Vaccine 13:1697-1705, 1995 ("Fouts").

Claims 1 and 4-6 were rejected under 35 U.S.C. § 103 additionally in view of Rock, U.S. Pat. No. 5,869,057 ("Rock"); Sztein et al., J. Immunol. 155:3987-3993, 1995 ("Sztein"); Vogelstein et al., U.S. Pat. No. 6,054,570 ("Vogelstein"); Chada et al., U.S. Pat. No. 5,736,388 ("Chada"); or Frankel et al., U.S. Pat. No. 6,099,848 ("Frankel").

REMARKS

Summary of the invention

In order to facilitate the examiner's understanding of the nature and character of the invention in more detail, applicants enclose the scientific publication Darji et al., Cell, 91:765-775 (1997) (Exhibit A) authored by the inventors of the present application. This publication in a highly regarded peer reviewed journal parallels the present application, which shows for the first time the successful genetic immunization of vertebrates using an attenuated *Salmonella* strain comprising a eukaryotic expression vector. The point of the invention is not to elicit an antibody response to a protein expressed by the *Salmonella*. Rather, the invention is genetic immunization using genes or gene fragments that are expressed by the host receiving the vaccine. In contrast to the art documents cited by the examiner, in which the *Salmonella* carried a vector containing a prokaryotic promoter, the vector in the vaccine used by applicants harbors a eukaryotic promoter (CMV immediate early promoter) that allows expression of the gene in a eukaryotic host cell.

1. The rejections under 35 U.S.C. § 112, second paragraph

Applicants have amended claim 9 as suggested by the examiner at page 4 of the official action. The rejections of claims 1, 9, 10, and 23 and claims dependent thereon at pages 6-7 of the official action are mooted by the amendments herein.

One of ordinary skill in the art would clearly know the difference between a polypeptide and a protein; however, applicants have removed this language from the claims in order to expedite prosecution.

2. The rejections under 35 U.S.C. § 112, first paragraph

The rejection of claim 1 and all claims dependent therefrom as assertedly containing new matter is mooted by applicants' amendment of the claim to delete the recitation of "polypeptide protein and/or antigen".

The rejection of claim 1 and all claims dependent therefrom as assertedly containing new matter because of the phrase "carried by the vector" is mooted by deletion of this language. However, applicants respectfully point out that the term "vector" is supported in many places throughout the specification, including at page 2, lines 19-22, page 3, line 29 through page 4, line 1, page 4, line 22, page 13, line 6 and page 14, line 20.

The rejection of claim 18 as assertedly containing new matter because of the phrase "IgG1, IgG2 and/or IgGA antibodies" should be withdrawn. It was apparently the examiner's

position that the specification only supported the "and", not the "or." However, the examiner's position is unsupported by the specification. The description of the various antibody responses occurs in completely separate sentences at page 7. Moreover, the IgG1 and IgG2 responses are discussed independently of each other at page 13, lines 15-28, and without discussion of the IgA response. Thus the specification clearly conveys to one of ordinary skill in the art that the specific types of antibody responses are contemplated individually in the alternative.

3. The rejections under 35 U.S.C. § 102

None of the cited art, Yang, Tite, Verma or Fouts, discloses an attenuated *Salmonella* strain which comprises a eukaryotic expression vector that allows efficient expression of the heterologous gene in the eukaryotic host which has been immunized with the *Salmonella*. All of the cited art merely disclose Salmonella strains bearing heterologous genes under the control of a prokaryotic promoter. The ability of the immunized eukaryotic host to express the gene is an important point because that is the mechanism by which the genetic immunization occurs. Applicants' experimental data at pages 8-10 showed that the immune response generated by the vaccine was due to the *in vivo* transfer of the gene and expression of the gene by the mice, not due to the expression of the gene by the *Salmonella*.

The examiner's repeated statements that the vectors in the various prior art attenuated *Salmonella* strains were capable of expressing the heterologous gene in the vertebrate host are incorrect. Applicants are unaware that significant expression of a gene by a prokaryotic promoter in a eukaryotic host is possible.

Thus, because none of the cited art discloses an attenuated Salmonella strain comprising a eukaryotic expression vector (under control of a eukaryotic promoter), the claimed invention is not anticipated and the rejection should be withdrawn.

4. The rejections under 35 U.S.C. § 103

None of the secondary references cited by the examiner, Rock, Sztein, Vogelstein, Chada or Frankel, corrects the glaring deficiency noted above under section 3. Sztein describes oral immunization of humans with *Salmonella* strains that have not been transformed with expression vectors at all, much less the type described in the claim. Frankel deals with immunization by *Listeria*.

Applicants note that Chada and Vogelstein do not deal with attenuated bacterial strains, let alone attenuated Salmonella strains for the vaccination of vertebrates. Vogelstein teaches the selective expression of desired genes in cells expressing oncogenes. Chada merely teaches

bacterial phage-mediated gene transfer systems capable of transfecting eukaryotic cells. Thus, Chada and Vogel stein are not properly combined with the other references cited by the examiner because they are from a different field and furthermore because the examiner has not shown where in the art such a combination is suggested. Moreover, even if the references are improperly combined, the combination does not teach applicants' claimed invention.

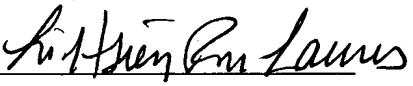
Thus, the cited references do not render obvious claim 1 or claims dependent thereon.

CONCLUSION

For the foregoing reasons, each of claims 1-6, 9, 10 and 17-23 is believed to be in condition for allowance. Accordingly, the examiner is respectfully requested to withdraw the outstanding rejections of the claims and to pass this application to issue.

Dated: December 10, 2004

Respectfully submitted,

By 

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Oral Somatic Transgene Vaccination Using Attenuated S. typhimurium

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Summary

An attenuated strain of S. typhimurium has been used as a vehicle for oral genetic immunization. Eukaryotic expression vectors containing truncated genes of ActA and listeriolysin—two virulence factors of Listeria monocytogenes—have been used to transform S. typhimurium aroA. Multiple or even single oral immunizations with such transformants induced excellent cellular and humoral responses. In addition, protective immunity was induced with listeriolysin transformants. The quality of the responses suggested a transfer of plasmid DNA from the bacterial carrier to the host. Such transfer was unequivocally shown in vitro with primary peritoneal macrophages. We describe a highly versatile system for antigen delivery, identification of protective antigens for vaccination, and efficient generation of antibodies against the product of open reading frames present on virtually any DNA segment.

Introduction

The design of efficient vaccines against infectious diseases remains a major challenge in medical science. Low cost, noninvasive administration, life-long protection by single doses combined with ease of preparation, storage, and transport are desirable goals to be achieved. In this respect, live, attenuated bacterial carriers that express heterologous antigens are attractive vehicles for the oral delivery of vaccines. This type of delivery should result in a broad spectrum of both mucosal and systemic immune responses. While oral subunit vaccines usually need to be coadministered with adjuvant proteins, such as cholera toxin, to evoke an effective immune response (Brown et al., 1987; Flynn et al., 1990), live replicating vectors produce their own immunomodulatory factors (e.g., cell-wall components) in situ, which also constitutes an advantage over other forms of administration such as microencapsulation. Moreover, the use of the natural route of entry could prove to be of benefit since many bacteria, like Salmonella, egress from the gut lumen via the M cells of Peyer's Patches (Neutra et al., 1996; Siebers and Finlay, 1996) and migrate eventually into lymph nodes and spleen, thus allowing targeting of vaccines to inductive sites of the immune system.

Genetic immunization has recently provided a promising new approach to vaccination (for review, see Donnelly et al., 1997). Isolated plasmid DNA—introduced into muscle or skin of the host—leads to expression of antigen in the host cells when transcription is driven by eukaryotic control elements. This has led to B- and T-cell stimulation and to protective responses. How these responses are generated still remains unclear. Muscle cells apparently express low levels of MHC class I, but lack MHC class II and costimulatory molecules. Although it is not known which cells function as antigen-presenting cells (APCs) under these circumstances, it is likely that resident dendritic cells or macrophages capture the antigen and migrate to lymph nodes and spleen to stimulate $CD4^+$ and $CD8^+$ T cells. Indeed, antigen-expressing dendritic cells have been observed following genetic immunization via the skin using a gene gun (Condon et al., 1996). It is not known whether DNA is also transferred directly into dendritic cells when plasmids are applied to muscle tissue.

Several advantages of genetic immunization over conventional vaccination have been observed. The DNA can be detected for a considerable period of time and thus acts as a depot of antigen (Zhu et al., 1993). Sequence motifs in some plasmids are immunostimulatory and can function as adjuvants (Krieg et al., 1995; Messina et al., 1991; Yamamoto et al., 1992). Coexpression of cytokines enhances the response and offers the possibility of modulating the induction of an immune response into a desired direction (Xiang and Ertl, 1995; Geissler et al., 1997; Kim et al., 1997). Despite this, several obstacles need to be overcome before general applicability can be achieved.

If it were possible to deliver plasmids for genetic immunization with an attenuated bacterial carrier, the advantages and versatilities of both systems could be combined. In addition, the natural route of administration would deliver DNA to cell types that have specifically evolved to induce immune responses. Salmonella spp. are particularly suited for this purpose because of the extensive knowledge on the genetics and physiology of many strains. A large body of documentation already exists pertaining to their utility as heterologous antigen carriers capable of inducing protective immune responses (Newton et al., 1989; Fairweather et al., 1990; reviewed by Chatfield et al., 1994; Roberts et al., 1994). Safe attenuated strains of Salmonella are available and are already in use as vaccines in man and farm animals (Germanier and Fürer, 1975; Hassan, 1996; Steinbach, 1996; Fox, 1997). Finally, recombinant plasmids constructed in laboratory strains of E. coli can be directly introduced into salmonellae without further manipulation.

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Here, we report that orally administered attenuated Salmonella typhimurium aroA bacteria carrying plasmids containing the coding sequences of β-galactosidase (β-gal) of Escherichia coli , or truncated forms of ActA or listeriolysin of Listeria monocytogenes, each under the control of an eukaryotic promoter, induce efficient humoral and cellular immune responses. The strength and kinetics of the responses is only compatible with the interpretation of a transfer of the expression plasmid from the Salmonella carrier to the nucleus of APCs of the host. β-galactosidase activity was detectable 5 weeks after administration of the oral vaccine. In addition, in vitro experiments with mouse primary macrophages demonstrated an efficient transfer of plasmid DNA from attenuated bacteria into the nucleus of the phagocytic host cells.

Results

To achieve genetic immunization with a live, attenuated bacterial carrier, three plasmids based on the commercially available plasmid pCMVβ were used. This plasmid contains the structural gene of β-gal under the control of the human CMV immediate-early promoter and includes a splice donor and two splice acceptor sites between the promotor and the structural gene. For studies examining the efficiency of the immune response against pathogens, the β-gal gene was substituted by genes encoding two virulence factors of Listeria monocytogenes: a truncated gene encoding a nonhemolytic variant of the listeriolysin protein (pCMVhly) consisting of amino acids (aa) 26–482, and a truncated gene of the membrane protein ActA (pCMVactA) encoding aa 31–613, were used. The S. typhimurium aroA strain SL7207 was transformed with these three plasmids, and groups of mice were orally immunized by feeding 10^9 organisms to each mouse per immunization. This dose was found to be optimal (data not shown). None of the mice showed any overt signs of illness during immunization.

Induction of a Strong T-Cell Response by Immunization with Salmonellae Carrying Eukaryotic Expression Vectors

These experiments were based on the working hypothesis that orally administered S. typhimurium aroA would result in uptake of the bacteria by macrophages and/or dendritic cells, with concomitant activation by bacterial endotoxin. After a few rounds of division, intracellular bacteria would die because of their inability to synthesize essential aromatic amino acids. During this process, plasmids would be released and transferred into the cytosol and the nucleus of the infected cells. Eventually, the encoded genes would be expressed by host APCs.

The first prediction of this hypothesis is that there should be a strong induction of cytotoxic CD8 T cells, since antigen would be expressed in the cytosol, the cellular compartment responsible for MHC class I presentation. To this end, two kinds of experiments were performed. Mice were either orally infected once with recombinant salmonellae and their cytotoxic T-cell responses followed for several weeks by testing their spleen cells directly ex vivo (data not shown) or after one restimulation in vitro. Alternatively, mice were orally immunized four times at 2 week intervals and the course of the cytotoxic response was examined. Figure 1 demonstrates that a strong and specific CD8 T-cell response was elicited with the orally administered Salmonella carrying eukaryotic expression plasmids. Mice immunized with the truncated gene of listeriolysin elicited a response only toward targets sensitized with the immunodominant peptide comprising aa 91–99 of listeriolysin (LLO) and not against targets sensitized with soluble hen egg lysozyme (HEL) or a control peptide (Figure 1A). Similarly, spleen cells from mice immunized with Salmonella carrying the ActA expression plasmid could only respond to ActA (Figure 1D). To reveal the cytotoxic response against ActA, we exploited the pore-forming activity of listeriolysin. This activity of listeriolysin allows the introduction of soluble passenger proteins into the cytosol of target cells (Darji et al., 1995a, 1997). Stimulators and target cells were therefore sensitized with a mixture of soluble ActA and LLO. A specific response was observed only when the combination of ActA and LLO was used. No response was found when LLO alone was tested. These responses were specific for the plasmid-encoded antigen during the entire time period indicated in Figures 1B & 1C and 1E & 1F, and were also observed when the responses of mice immunized with Salmonella harboring the β-gal control plasmid was studied (data not shown).

The kinetics of the responses indicated that even a single dose elicited a strong cytotoxic T-cell response that peaked 5 weeks after immunization and then slowly declined (Figures 1C and 1F). On the other hand, the response was still rising even at the end of the observation period, i.e., 5 weeks after the last booster dose, in mice that had received four doses (Figures 1B and 1E). Thus, a strong cytotoxic response was observed with Salmonella as a vehicle for genetic immunization.

Genetic immunization in addition usually evokes a CD4 helper-T-cell response (Donnelly et al., 1997). Therefore, cells from spleens and mesenteric lymph nodes of the same mice used above were tested for their proliferative response against soluble proteins. This type of response is mainly due to presentation of antigen via MHC class II molecules and is carried out by CD4 T cells. As shown in Figure 2, a strong and specific helper-T-cell response, which paralleled the cytotoxic response, was observed when eukaryotic expression plasmids carried by salmonellae were used for immunization (Figures 2A and 2D). As with the CD8 response, a single dose was sufficient for a good response that was still increasing at the end of the observation period, regardless of whether listeriolysin or ActA was used as antigen (Figures 2C and 2F). Four consecutive immunizations, however, resulted in an even stronger response that appeared to be long-lasting since it apparently was still increasing 5 weeks after the last booster (Figures 2B and 2E). Similar results were obtained with Salmonella carrying the control plasmid expressing β-gal (data not shown). Analysis of the supernatants of the in vitro cultures revealed production of

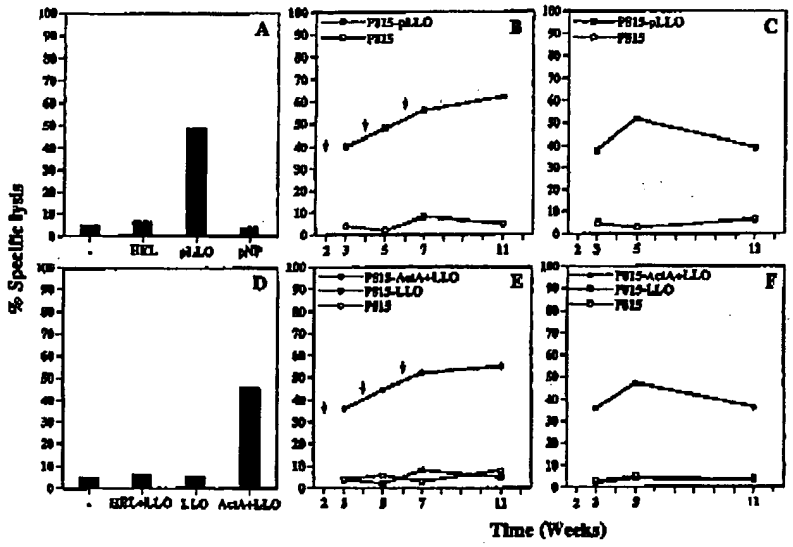

Figure 1. Induction of Cytotoxic T Cells in Mice Orally Immunized with S. typhimurium aroA Carrying Eukaryotic Expression Plasmids Encoding Listeriolysin or ActA Fragments

Mice were immunized with 10^9 bacteria either four times at 2 week intervals (A, B, D, and E) or once (C and F) with Salmonella carrying pCMVhly (A–C) or pCMVactA (D–F). Spleen cells were restimulated once in vitro with a synthetic peptide comprising aa 91–99 of listeriolysin (A–C) or with a mixture of purified ActA and hemolytically active listeriolysin, which results in the class I presentation of ActA due to the pore-forming activity of listeriolysin (Darji et al., 1995a, 1997). Restimulated T cells were tested with radiolabeled P815 target cells at an effector-to-target ratio of 10:1. (A) Specificity of the anti-listeriolysin cytotoxic response. Target cells were sensitized with hen egg lysozyme (HEL), peptide aa 91–99 of listeriolysin (pLLO), or control peptide aa 147–155 of nucleoprotein of influenza virus (pNP). Displayed is the experiment with spleen cells from week 5 shown in (B). Similar specificity was observed at all other time points.

(B) Kinetics of the cytotoxic response of mice immunized four times with pCMVhly. The arrows indicate the booster immunizations.

(C) Kinetics of the cytotoxic response of mice immunized once with pCMVhly.

(D) Specificity of the anti-ActA cytotoxic response. Target cells were sensitized with a mixture of ActA and listeriolysin (ActA + LLO), HEL and listeriolysin (HEL + LLO), or listeriolysin alone (LLO). Displayed is the experiment with restimulated spleen cells from week 5 shown in (E). Similar specificity was observed at other time points and including other synthetic peptides of various sources.

(E) Kinetics of the cytotoxic T-cell response in mice immunized four times with pCMVactA. Arrows indicate booster immunizations.

(F) Kinetics of the cytotoxic T-cell response in mice immunized once with pCMVactA. The specificity of the cytotoxic response was further assessed by testing the spleen cells of mice immunized in a similar way with pCMVβ (β-gal) on target cells sensitized with pLLO, ActA plus listeriolysin, or a β-gal-expressing transfectant of P815 (data not shown). Similarly, a specific cytotoxic T-cell response was observed against β-gal, but the kinetic was not followed as systematically as for the two other antigens.

IFNγ by these T cells. No IL-4 was found, which suggests that such an immunization scheme mainly induces a Th1 or inflammatory type of T-helper response.

Induction of Specific Antibodies by Immunization with Salmonellae Carrying Eukaryotic Expression Vectors

Pooled sera of the groups of mice used above were tested for the presence of specific antibodies. Clearly, in addition to a cytotoxic and helper-T-cell response, immunization with salmonellae carrying eukaryotic expression plasmids induced strong and specific antibody responses as revealed by ELISA (Figures 3A and 3B) or immunoblot (data not shown). Again, a single immunization was sufficient for a good response that peaked 4 weeks after the administration of the bacteria and then declined in the same way as seen for the cytotoxic response. Four immunizations did not increase the antibody titer significantly but probably induced a longer lasting response since the plateau of antibody titers was maintained even at the end of the observation period (Figures 3A and 3B).

Analysis of the IgG subclass distribution in the immune sera of individual mice at week 11 indicated a high concentration of IgG2a, while the concentrations of IgG2b and IgG3 were negligible (Figures 3C and 3D). This is in agreement with the finding that only IFNγ and no IL-4 could be detected in the supernatants of the restimulated T-helper cells. However, IgG1 was also observed at high concentrations in the immune sera. This subclass is found when Th2-helper cells participate in the immune response (Mosmann and Coffman, 1989), which indicates that under our experimental conditions Th2 cells might also be induced but were not revealed in the in vitro T-cell assay. In addition, serum IgA antibodies were evoked by this immunization schedule (data not shown).

Taken together, the results presented in Figures 1–3

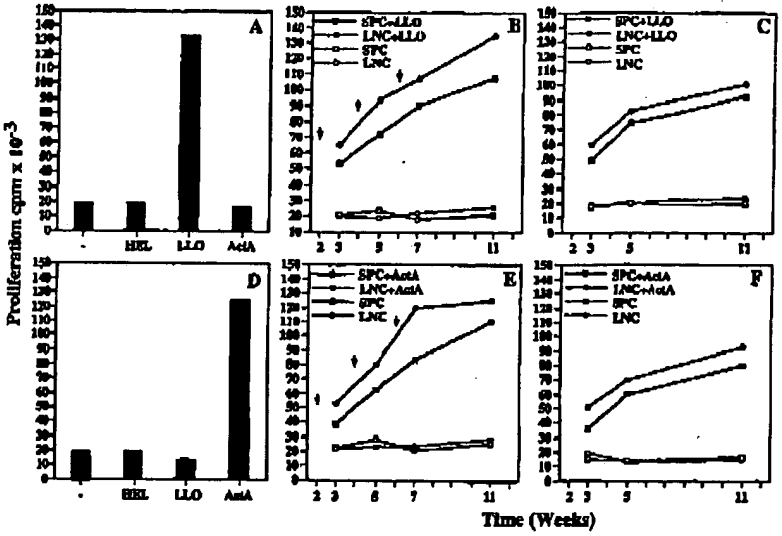

Figure 2. Induction of Helper-T Cells

Spleen (SPC) and lymph node cells (LNC) from the same mice tested for cytotoxic T-cell responses described in Figure 1 were tested for T-helper responses. Mice were immunized either four times (A, B, D, and E) or once (C and F) with 10^8 Salmonella carrying pCMVhly (A–C) or pCMVactA (D and F) and restimulated in vitro. After 2 days, proliferation was tested by incorporation of ^{3}H-Thymidine.

(A) Specificity of the proliferative response of spleen cells from mice immunized with pCMVhly. T cells tested were the same as those displayed in (B) at week 11. Similar results were obtained at other time points.

(B) Kinetics of the proliferative response of spleen and lymph node cells from mice immunized four times with pCMVhly. Arrows indicate the booster immunizations.

(C) Kinetics of the proliferative response of spleen and lymph node cells from mice immunized once with pCMVhly.

(D) Specificity of the proliferative response of spleen cells from mice immunized four times with pCMVactA. T cells tested were the same as those displayed in (D) at week 11. Similar results were obtained at other time points.

(E) Kinetics of the proliferative response of spleen and lymph node cells immunized four times with pCMVactA. Arrows indicate booster immunizations.

(F) Kinetics of the proliferative response of spleen and lymph node cells from mice immunized once with pCMVactA. Spleen and lymph node cells from mice immunized with pCMVβ (β-gal) never reacted with either listeriolysin or ActA but could respond to restimulation with β-gal (data not shown).

show that immunization with S. typhimurium aroA carrying eukaryotic expression vectors can evoke responses in all three specific effector compartments of the immune system, namely, cytotoxic CD8 T cells, CD4 T cells, and antibodies. The response in the T-helper compartment was strongly biased toward a Th1- or inflammatory T-helper response.

Protection against Lethal Doses of L. monocytogenes

The strong responses observed above, in particular that of cytotoxic T cells, suggested that mice immunized in this way should be protected from a lethal dose of L. monocytogenes. Therefore, 90 days after the first immunization or 48 days after the fourth immunization (where applicable) mice were challenged intravenously with a dose of bacteria corresponding to 10 × LD_{50}. Figure 4 shows that animals that were immunized four times consecutively with salmonellae harboring a eukaryotic expression vector that encodes truncated LLO were completely protected (Figure 4A). Animals that had received only a single vaccination were significantly protected, and 60% of the animals were still alive at the time of termination of the experiment. All animals that were immunized with salmonellae that carried the β-gal control plasmid were not protected and died within four days. Perhaps surprisingly, immunizations with salmonellae carrying the ActA expression plasmid did not result in protection, although strong cytotoxic and helper-T-cell responses could be demonstrated in such mice, indicating that the immunization had been successful (data not shown). Thus, the listerial membrane protein ActA is not a protective antigen.

Evidence for Transfer of the Expression Plasmid from the Carrier Salmonellae to Host Cells In Vivo

We were concerned that a weak activity of the eukaryotic promoter in the host bacteria or a cryptic prokaryotic promoter in the plasmid might have resulted in expression of the antigens in the bacterial carrier, thereby eliciting the potent immune response. In fact, the recombinant salmonellae harboring the pCMVβ exhibited low β-gal activity (2.5 U) compared to the parental strain. To assess this possibility, we immunized mice with a recombinant Salmonella strain that produced more than 100-fold higher levels (334 U) of β-gal enzymatic activity.

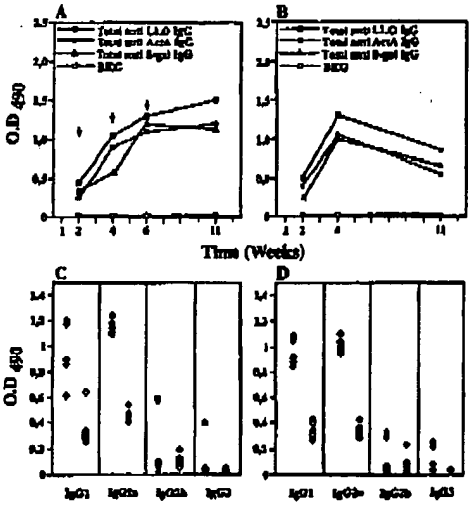

Figure 3. Kinetics and Subclass Distribution of Specific Serum IgG

Sera from the same mice tested for cytotoxic and proliferative T-cell responses described in Figures 1 and 2 were used and assayed in specific ELISAs. Mice were immunized four times (A) or once (B) with pCMVhly, pCMVactA, or pCMVβ, respectively, and pooled sera were tested for antigen-specific serum IgG. To assess specificity, all sera were tested on all three antigens. Reactivity was only observed against the immunizing antigen (data not shown). Identical results were obtained by immunoblotting using the same antigens (data not shown). The subclass distribution 11 weeks after the first immunization was determined from the sera of individual mice immunized four times (closed symbols) or once (open symbols) with either pCMVhly (C) or pCMVactA (D).

A single vaccinating dose using these bacteria did not elicit any measurable T-cell or antibody responses (Figures 5A–5C). Repeated vaccination, however, resulted in a weak cytotoxic T-cell response, detectable after in vitro restimulation, although it barely reached the strength of the response observed using a single immunization with salmonellae harboring the β-gal eukaryotic expression plasmid (Figure 5A). Neither a CD4 T cell nor an antibody response was observed, even after repeated oral immunization with salmonellae constitutively expressing β-gal (Figures 5B and 5C).

As a result of the aroA mutation, bacteria do not appear to survive very long since live bacteria could never be recovered from immunized animals at the various time points examined. Nevertheless, β-gal activity was detected in adherent cells—most likely macrophages—from the spleen of mice 5 weeks after oral administration of salmonellae harboring the eukaryotic β-gal expression plasmid, which suggests that plasmid transfer to the eukaryotic cell had taken place (data not shown). To extend this observation, we injected salmonellae carrying the pCMVβ vector into the peritoneum of mice and harvested the peritoneal exudate after 1 hr. Subsequently, cells were cultured overnight in the presence of tetracycline to inhibit bacterial protein synthesis and then stained for β-gal activity. β-gal activity was observed in a large number of macrophage-like cells. The

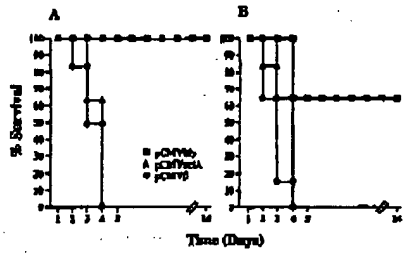

Figure 4. Protective Immune Response after Oral Genetic Immunization

Groups of six mice were immunized four times at 2 week intervals (A) or only once (B) with Salmonella carrying pCMVhly, pCMVactA, or pCMVβ and challenged intravenously with a lethal dose of 5 × 10^4 L. monocytogenes EGD (10 × LD_{50}). Mice that had been immunized only once with pCMVhly showed signs of distress one day after injection and two died 3 days later, whereas the remaining four recovered and survived in good condition until the experiment was terminated 2 weeks later.

staining was diffuse and clearly not restricted to the endocytic vesicles in which salmonellae usually reside. This suggests that plasmid DNA was efficiently transferred from dying salmonellae to host cells.

DNA Transfer from S. typhimurium aroA to Mammalian Host Cells in Vitro

To obtain direct evidence that DNA transfer from the bacterial carrier to the mouse macrophages can take place, primary peritoneal macrophages were infected in vitro for 1 hr with salmonellae harboring the β-gal expression plasmid (pCMVβ), after which gentamicin was added to kill remaining extracellular bacteria. Tetracycline was added 4 hr later to kill any viable intracellular bacteria. After overnight incubation, cells were stained for β-gal activity. Up to 30% of the adherent, macrophage-like cells exhibited β-gal activity despite the continuous presence of tetracycline that blocks bacterial protein synthesis (Figure 6).

To rigorously demonstrate that β-galactosidase was synthesized de novo by the host cell, and not by the bacterial vector, two types of experiments were performed. First, adherent peritoneal cells were infected and treated as described above. After overnight incubation, RNA was extracted. If the plasmid had indeed been transferred and transcribed in the nucleus of the host cell, RNA splice products derived from the splice donor and acceptor sites within the vector should be demonstrable. By means of RT-PCR with a primer pair that hybridizes to sequences on either side of the small intron, a PCR product was obtained that corresponded to one of the expected splice products (Figure 7A). The identity of this product was confirmed by DNA sequencing (data not shown). Second, biosynthetic labeling of proteins in the presence of tetracycline should only allow translation of mRNA produced by the eukaryotic host cells. Adherent peritoneal cells were infected as described and were pulsed for 30 min with ^{35}S-methionine after 4, 24, or 48 hr in the absence or presence of tetracycline. At 4 hr, no β-gal could be observed by immunoprecipitation, even in the absence of tetracycline where

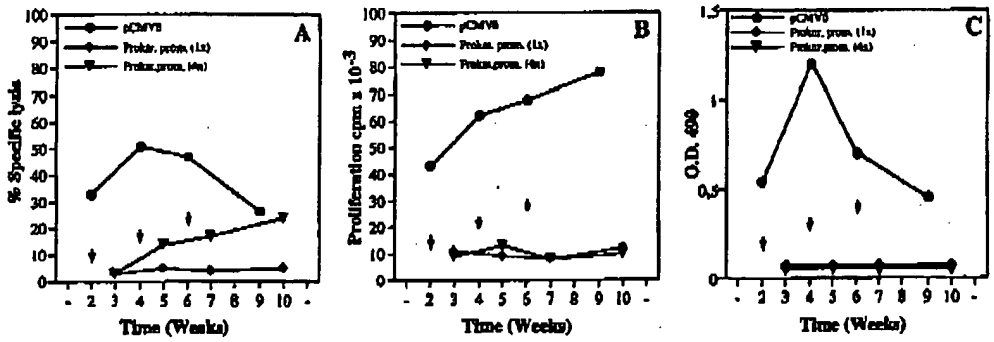

Figure 5. Comparison of Orally Induced Immune Responses Elicited with Salmonella Harboring Prokaryotic or Eukaryotic β-Gal Expression Plasmids

Mice were immunized with Salmonella harboring either the eukaryotic expression plasmid pCMVβ or the plasmid pAH97 that constitutively expressed β-gal from the Pr and Ps promotors of *xylS* of Pseudomonas putida. Bacteria harboring the eukaryotic vector were administered orally once (closed circles), whereas bacteria expressing β-gal under the control of the prokaryotic promotor were administered either once (closed diamonds) or four times with 2 week intervals (closed upside-down triangles). The arrows indicate the time of booster immunizations.
(A) Cytotoxic response of restimulated spleen cells tested at an effector-to-target ratio of 10:1. The β-gal-expressing transfectant P19.1 was used as target in the JAM assay.
(B) Proliferative helper-T-cell response of spleen cells with isolated β-gal as antigen.
(C) Antibody response against β-gal from pooled sera measured by ELISA. Data displayed in (A)–(C) were obtained with cells or sera from the same mice. All assays were performed as described in Figures 1–3.

bacterial products should have been labeled (Figure 7B). Thus, transfer of plasmid DNA and eukaryotic expression had not yet taken place. However, β-gal could be immunoprecipitated after 24 or 48 hr of incubation, even when tetracycline was continuously present during both the incubation and labeling period. Preincubation of the anti-β-gal antibody with an excess of unlabeled β-gal demonstrated the specificity of the immunoprecipitation (Figure 7B, lane 10). This clearly indicates that the precipitated β-gal was produced by the infected mammalian host cell itself and not by the bacterium that had originally carried the expression plasmid. Thus, a transfer of the plasmid from salmonellae to the host cell must have taken place.

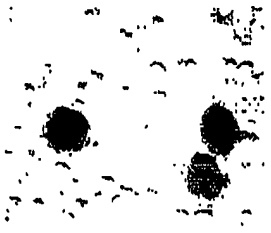

Figure 6. Expression of β-Gal Activity in Peritoneal Exudate Cells

Freshly isolated peritoneal exudate cells (PECs) were allowed to adhere for 2 hr and were infected at an MOI of 10 for 15 min with Salmonella bearing pCMVβ in antibiotic-free medium. Following a wash and addition of gentamycin to kill extracellular bacteria, incubation was continued for 3–4 hr at 37°C, and tetracycline was then added to the medium to kill any viable bacteria by blocking their protein synthesis. After 24 hr, expression of β-gal activity in up to 30% of the adherent cell population was observed. Only macrophage-like cells expressed enzymatic activity. The small cells found in the cultures most likely represent nonadherent lymphocytes that were not removed in this particular experiment. Staining the cells at the time of addition of tetracyclin did not reveal any enzymatic activity.

Discussion

The transfer of eukaryotic expression plasmids from attenuated enteric bacteria into the nucleus of host cells has recently been demonstrated. Auxotrophic mutants of Shigella and E. coli that express the invasin of Shigella can introduce eukaryotic expression plasmids into host cells (Courvalin et al., 1995; Sizemore et al., 1995). Given that both bacteria are capable of escaping from the phagolysosome into the cytosol of the host cell, it follows that lysis of bacteria in this compartment would allow transfer of the released plasmid DNA into the nucleus. Transfer of plasmid from intracellular pathogens such as Salmonella would be harder to imagine, as these bacteria are generally retained within vacuoles of the infected host cell. Indeed, only a "low efficiency" of plasmid transfer into a macrophage cell line using attenuated Salmonella had been reported (Sizemore et al., 1995). Our initial experiments using several macrophage cell lines confirmed this observation (data not shown).

However, the kinetics and strength of the immune response elicited by administration of Salmonella carrying eukaryotic expression vectors, which we observed here, suggested that a plasmid transfer can take place in vivo. We therefore decided to investigate primary macrophages isolated from the peritoneum of mice. Compelling evidence for a transfer of plasmid DNA from Salmonella to the host cell in vitro was obtained. Both the splicing of RNA, and protein synthesis in the presence of tetracycline, are only possible if the gene is expressed by the eukaryotic host cell. Evidence that

Figure 7. The Eukaryotic Host Cells Transcribe and Translate the β-Gal Gene Derived from S. typhimurium aroA Harboring the Expression Plasmid

(A) RNA derived from PECs 24 hr after infection with Salmonella carrying pCMVβ was analyzed by RT-PCR. A primer pair that borders the splice donor and acceptor sites downstream of the promotor was used. In lane 2, a band of 186 bp (indicated by the arrow) was detected. DNA sequencing identified this fragment as a splice product. The stronger 227 bp fragment seen in this lane is either due to carryover of DNA into the RNA preparation or due to inefficient splicing. Lane 1 shows the untreated macrophage control, and the lane marked (M) contains the molecular size marker used. Since the signal of the splice product was extremely weak, we inverted the colors from white to black. This resulted in an increase of contrast and allowed the visualization of the splice product on the graph.
(B) PECs were infected with pCMVβ carrying Salmonella as described and, after incubation for various lengths of time, biosynthetic labeling was performed with ^{35}S-methionine in the presence or absence of tetracycline, followed by immunoprecipitation with β-gal-specific monoclonal antibodies. Controls: (1) BHK cells; (2) BHK cells transfected with β-gal (positive control). Infected PECs: (3) incubated 4 hr postinfection without tetracycline; (4) incubated 4 hr postinfection with tetracycline during labeling; (5) incubated 4 hr postinfection with tetracycline during incubation and labeling; (6) incubated 24 hr postinfection without tetracycline; (7) incubated 24 hours postinfection with tetracycline during labeling; (8) incubated 24 hr postinfection with tetracycline during incubation and labeling (9) incubated 48 hr postinfection without tetracycline; (10) incubated 48 hr postinfection with tetracycline during labeling. In this case, a 100-fold excess of unlabeled β-gal over the precipitating antibody was added to the lysate before immunoprecipitation; (11) incubation of 48 hr postinfection with tetracycline during labeling; (12) incubation 48 hr postinfection with tetracycline during incubation and labeling. No specific band was observed after 4 hr of incubation under any conditions. However, after allowing 24 hr or more for a DNA transfer and expression to occur, a specific band for β-gal (indicated by the arrow) can be observed.

transfer of the expression vector in vivo is responsible for induction of the strong immune response observed was also obtained. Although viable Salmonella could not be isolated one week after the last booster, β-gal activity was observed in some adherent cells 5 weeks later, which suggests that β-gal expression cannot be due to residual surviving Salmonella. It is, however, intriguing how such antigen-expressing cells can coexist in the presence of specific cytotoxic T cells.

A pathway that permits transfer of proteins from endocytic vesicles into the cytosol of some cell types, including macrophages, has been described (Reis de Sousa and Germain, 1995; Norbury et al., 1995). Whether or not such a pathway is also responsible for the transfer of nucleic acids reported here remains to be clarified. The fact that plasmid transfer with Salmonella was only observed with primary macrophages and not with cell lines suggests the presence of a transport pathway that only operates efficiently in primary cells.

Strong cytotoxic and protective responses to antigens delivered by Salmonella have only been reported with Salmonella recombinants that secrete the antigens; no comparable responses have been reported using Salmonella that constitutively express nonsecreted heterologous proteins (Hess et al., 1996). Turner et al. (1993) have demonstrated that high doses of recombinant bacteria expressing intracellular protein are required to induce CD8 T cells. Although induction of specific antibodies has been described under some experimental conditions (Guzmán et al., 1991; Walker et al., 1992), no antibody response was observed under the experimental conditions used by Turner et al. (1993). This was confirmed by our own results (Figure 5). We therefore consider it to be highly unlikely that the strong cytotoxic and helper-T-cell responses and specific antibody production are the results of any fortuitous expression of the antigens in the Salmonella carrier.

The strength of the immune response observed, even with a single immunization, indicates that transfer of DNA by bacterial carrier is probably superior to that achieved by direct application of isolated plasmid DNA into skin or muscle. This suggests that by using the natural port of entry of a pathogen, the expression vector is transferred into cell types that have evolved to efficiently induce an immune response. It is likely that the Salmonella carrier is taken up by macrophages and dendritic cells. Whether macrophages play a role during stimulation of naive T cells against bacteria is not clear, but dendritic cells are known to be highly efficient in priming resting T cells. Since the antigen is expressed in the cytosol of these cells, a strong cytotoxic T-cell response is to be expected.

The induction of strong helper and antibody response is puzzling and can presently only be speculated upon. Some cytosolic proteins can be efficiently presented by MHC class II molecules (Jaraquemada et al., 1990; Brooks and McCluskey, 1993). However, it would be very fortuitous if all three proteins used in the present study display this property. In any case, it could not explain the antibody responses that we observed.

Our interpretation of oral genetic immunization using attenuated salmonellae as carrier is schematically depicted in Figure 8. Salmonella enter the host via M cells in the intestine. The bacteria are taken up in the dome areas by phagocytic cells such as macrophages and dendritic cells. These cells are activated by the pathogen and start to differentiate and probably to migrate into lymph nodes and spleen. During this period, the bacteria die due to their attenuating mutation and liberate the plasmid-based eukaryotic expression vectors. The plasmids are then transferred into the cytosol either via a specific transport system or by endosomal leakage. Finally, the vector enters the nucleus and is transcribed, leading to antigen expression in the cytosol of the host cells. Specific cytotoxic T cells are induced by these activated APCs that lyse antigen-expressing cells. Free antigen or dying cells are taken up by other APCs, which in turn stimulate helper cells. Free antigen would also be responsible for the induction of an antibody response. Bacterial endotoxin and DNA sequence motifs of the

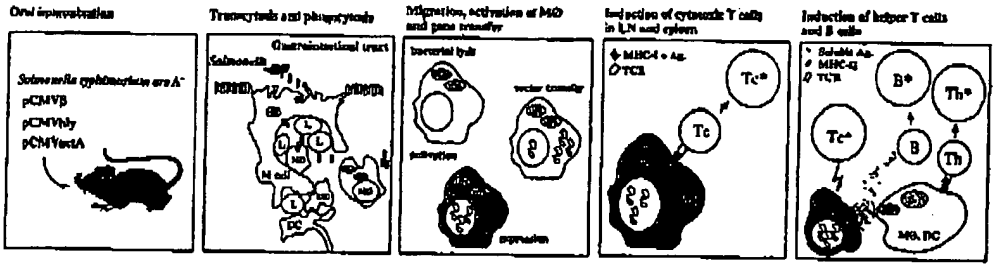

Figure 6. Schematic Representation of the Sequence of Events Assumed to Occur in Vivo after Oral Genetic Immunization with Attenuated S. typhimurium aroA

See text for further details.

vector may also function as adjuvant and could contribute to the strength of the responses observed.

The helper-T-cell response induced with this type of genetic immunization seemed strongly biased toward the Th1 type as indicated by IFN-γ production of restimulated T cells in vitro and the high titer of IgG2a in the humoral response (Mosmann and Coffman, 1989). This is not unexpected since bacteria usually induce inflammatory types of response. For many vaccination strategies, it is desirable to induce a Th1 response for protection against the corresponding pathogen. For example, strains of mice that respond with Th2 cells against Leishmania major do not clear the parasite and are not protected, whereas mice that mount a Th1 response are resistant (Sher and Coffman, 1992). On the other hand, induction of a Th2 type response or the conversion of a Th1 response into a Th2 response has been shown to be advantageous in inflammatory autoimmune diseases (Tian et al., 1996). Similarly, infections by nematodes might also require a Th2 response (Sher and Coffman, 1992). Since the bacteria are only being used as a vehicle to transfer the expression plasmids, and therefore play only a secondary role, it should be possible to manipulate the Th1 response. The induction of specific IgG1 suggests the presence of a Th2 component during the helper response that might be augmentable. Coexpression of the antigen together with certain cytokines or costimulatory molecules, or exploitation of antisense strategies to suppress costimulatory molecules, should make it possible to drive the responses more toward Th2.

Two well-characterized virulence factors were tested as antigens for protection against a lethal challenge with L. monocytogenes. Listeriolysin has been shown before to induce protection (Harty and Bevan, 1992; Hess et al., 1996). This was also true under our experimental conditions. Interestingly, even a single dose of salmonellae harboring the eukaryotic listeriolysin expression plasmid was sufficient to afford 60% of the mice protection against a lethal challenge. On the other hand, despite the strong ActA-specific cytotoxic, helper, and antibody responses induced by the pCMVactA vector, no protection was elicited. ActA is thus not a protective antigen in this system. The membrane protein ActA on challenge bacteria is obviously not available to the presentation mechanisms as long as the bacteria are alive (Darji et al., submitted) (e.g., extensive phosphorylation of the ActA protein by host kinases following infection may affect its ability to be processed). Nevertheless, the role of bacterial surface-bound proteins in protection can easily be addressed now using the salmonellae system for genetic vaccination.

The induction of a strong and specific antibody response that can be measured in ELISA and by immunoblot revealed additional benefits derived from the type of immunization described here. Our results suggest that it may be possible to insert practically any open reading frame into a eukaryotic expression plasmid and use Salmonella to deliver it to mice to raise high titer-specific polyclonal and possibly also monoclonal antibodies. This will facilitate the characterization of gene products where only sequence information is available. The possibility of genetic immunization with DNA fragments containing open reading frames will allow researchers to define the function of new gene products, provide novel serological reagents, and permit delineation and assess efficacies of protective antigens in vaccination protocols.

In conclusion, attenuated Salmonella that carry eukaryotic expression vectors can be used for genetic immunization via the oral route. The stimulation of cytotoxic and helper T cells as well as the induction of a strong antibody response provides a very versatile system for new immunization strategies. The strength of this approach also draws on the development of newer, more rationally attenuated salmonellae strains as well as technical advances in providing conditional and targeted eukaryotic expression by the infected host cell.

Experimental Procedures

Mice

6- to 8-week-old female BALB/c (H-2^d) mice were used.

Media, Reagents, and Antigens

Cells were cultured in RPMI/10% fetal bovine serum. Solid and liquid Luria Bertani medium (LB, Sambrook) was used to grow E. coli and S. typhimurium strains. Brain heart infusion broth or agar (BHI; Difco, Detroit, MI) was used to grow L. monocytogenes. Media were supplemented, where required with 100 μg/ml of ampicillin. Listeriolysin was purified as described (Darji et al., 1996b). Soluble ActA protein (aa 31–505) was purified from supernatants of recombinant L. monocytogenes.

Bacterial Strains and Plasmids

The E. coli strain XL1-Blue (Stratagene, Heidelberg, Germany) was used as a host during the cloning experiments and to propagate plasmids. The auxotrophic S. typhimurium aroA strain SL7207 (S. typhimurium 2337–66 derivative *hisG46*, DEL407 [*aroA*::Tn*10*(Tc-s)]), kindly provided by Dr. B. A. D. Stocker, Stanford, CA, was used as a carrier for the in vivo studies. L. monocytogenes strain EGD (serotype 1/2a; Chakraborty et al., 1992) was used for in vivo protection assays and for preparation of genomic DNA to clone *actA* and *hly* genes. The eukaryotic expression vector pCMVβ (Clontech, Palo Alto, CA) was used for cloning a truncated variant of *actA* or *hly*. For expression of β-gal in salmonellae, the plasmid pAH97 was used (Hoiseth et al., 1992). It contains the Pr and Ps promotor of the *xylS* gene of Pseudomonas putida and results in constitutive expression of β-gal (384 U) in the S. typhimurium aroA strain.

Recombinant DNA Techniques

DNA preparation, genetic manipulations, PCR, and transformation of bacteria were carried out according to standard protocols (Sambrook et al., 1982; Hanahan, 1983; O'Callaghan and Charbit, 1990).

Cloning of *actA* and *hly* into the Eukaryotic Expression Vector pCMVβ

For the construction of the eukaryotic expression vector pCMVactA, a 1.8 kb fragment encoding aa 31–613 of an ActA polypeptide without the membrane anchor (Domann et al., 1992) was amplified by PCR using primers: 5′-ATAAGAATGCGGCCGCCATGGCGACAGAT AGCGAAGATTCTAGTC-3′ and 5′-ATAAGAATGCGGCCGCTTACGT CGTATGGTTCCCTGGTTCTTC-3′. In a similar way, recombinant plasmid pCMVhly was constructed. A 1.4 kb fragment encoding a non-hemolytic variant comprising aa 26–482 of *hly* (Mengaud et al., 1988) was amplified using primers: 5′-ATAAGAATGCGGCCGCCAT GGATGCATCTGCATTCAATAAAGAAAATTC-3′ and 5′-ATAAGAAT GCGGCCGCTTATTTAGCGTAAACATTAATATTTCT CGCG-3′. PCR primers contain NotI flanking restriction sites (underlined). Start and stop codons were also introduced (ATG and TTA in bold). pCMVactA and pCMVhly were generated by replacing β-gal of pCMVβ by the PCR fragments.

Immunization and Challenge

Groups of 6–10 female BALB/c mice were fed with 30 μl of 10% sodium bicarbonate buffer containing 10^9 recombinant S. typhimurium aroA strain harboring one of the eukaryotic expression vectors or the prokaryotic β-gal expression plasmid pAH97 (Hoiseth et al., 1992). Mice received either a single immunization or four immunizations at 14-day intervals. Serum samples from both groups of mice were obtained on days −1, 7, 21, 35, and 63. Mice of each group were sacrificed at weeks 3, 5, 7, and 11 after the first immunization and tested for T-cell responses. For protection studies, immunized mice were challenged intravenously on day 90 with a lethal dose of 5×10^4 L. monocytogenes. Survival of mice was followed until day 14 post-challenge. All these experiments were performed at least two times.

CTL and Proliferation Assay

For the determination of induction of cytotoxic T cells, the JAM assay was performed (Matzinger, 1991). To test for LLO-specific cytotoxic T cells, P815 target cells were sensitized with 1 μg/ml of LLO peptide aa 91–99 (Parner et al., 1991). The ActA-specific cytotoxicity was revealed by sensitizing the radiolabeled P815 cells with a mixture of 1 μg/ml purified, hemolytically active LLO and 1 μg/ml of purified ActA protein for 30 min at RT. To measure the β-gal-specific cytotoxicity, P13.1—a P815 derivative transfected with the β-gal gene—was used as target cells (Rammensee et al., 1989). Proliferation of T cells were directly analyzed by ^{3}H-thymidine incorporation as described (Darji et al., 1995a).

ELISA

To evaluate the levels of specific immunoglobulins or IgG subclasses in serum, standard ELISA was employed with biotinylated goat anti-mouse Ig (Dianova, Hamburg, Germany) or directly conjugated goat anti-mouse IgG1, IgG2a, IgG2b, and IgG3 (Caltag Laboratories, CA).

Detection of β-Gal Activity

Expression of β-gal in host cells was monitored by incubating the fixed cells with the indicator substrate X-gal. Briefly, isolated peritoneal macrophages were allowed to adhere for 1–2 hr at 37°C in a 24-well plate in antibiotic-free medium. After removing the nonadherent cells and washing with antibiotic-free medium, S. typhimurium aroA harboring the eukaryotic expression vector pCMVβ were added to the cells at a MOI of 10 and incubated at 37°C for 15–30 min. Cells were washed again, and bacteria remaining extracellular were killed by addition of medium containing 50 μg/ml gentamicin. Following 4 hr of incubation at 37°C, 10 μg/ml of tetracycline was added to some of the cultures to block the intracellular bacterial multiplication, and incubation was continued for another 24 hr. After 2–3 washes with PBS, cells were fixed with acetone/methanol (1:1 v/v) and freshly prepared X-gal substrate (5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM $MgCl_2$ and 100 mg/ml X-gal in PBS) was added. After overnight incubation at 37°C, β-gal-expressing cells were detected by light microscopy. Quantitation of β-gal enzymatic activity in recombinant bacteria was determined according to standard procedures (Sambrook et al., 1982). Background (4U) was substracted from the experimental values.

RNA Isolation and RT–PCR

To test for expression of β-gal transferred into the eukaryotic host cells via Salmonella, the mRNA was probed for the presence of splice products derived from the splice donor and acceptors of the expression plasmid. To this end, PECs were infected in vitro at an MOI of 10 with S. typhimurium aroA harboring the eukaryotic expression vector pCMVβ and isolaton of RNA, and RT–PCR was performed according to standard protocols (Chomczynski and Sacchi, 1987; Wilks, 1989). PCR was performed for 35 cycles of 20 s 95°C, 30 s 60°C, and 30 s 72°C. The primer pair was designed in such a way that the presence of splice products should be indicated by a 180 bp and/or a 125 bp fragment. Primers used: SV40f, 5′-GGATCCGGTACTCGAGGAAC-3′; SV40r, 5′-GCTTTAGCAGGCTCT TTCG-3′.

Immunoprecipitations

5×10^6 adherent PECs were infected with ~5×10^7 S. typhimurium aroA harboring pCMVβ, incubated for 4 hr in medium with or without antibiotics, and left at 37°C for various periods of time. Cells were then starved 30 min in methionine-free medium and pulsed with 100 μCi [^{35}S]methionine for 2 hr. Immunoprecipitations were performed with anti-β-gal antibodies (Promega) and analyzed on 8% SDS-PAGE followed by fluorography. Into some samples, a 100-fold excess of β-gal protein was added before addition of anti-β-gal antibodies to determine the specificity of the precipitation.

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